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COMPOSITION FOR IVF

The present invention relates to a solid, chemically stable composition that can be used in connection with in vitro fertilisation or in vitro maturation (hereinafter designated IVF or IVM, respectively). More specifically, it relates to a container containing such a solid composition.

BACKGROUND OF THIS INVENTION

Several meiosis activation substances (hereinafter designated MAS) are known. When MAS are kept in a medium containing oocytes, the oocytes become more prone to fertilisation. However, a major problem with the use of MAS is that, usually, they have a very low solubility and low chemical stability under the conditions at which they are to be used.

SUMMARY OF THIS INVENTION

One object of this invention is to develop a composition containing MAS or a derivative thereof that can be dissolved in an aqueous medium in a sufficient concentration to be used for IVF or IVM.

Another object is to develop a composition containing MAS or a derivative thereof which can be dissolved in an aqueous medium without any physical influence such as heating, stirring, or ultrasound treatment.

A third object is to develop a composition containing MAS or a derivative thereof which has a sufficient chemical and physical stability at the conditions under which it is stored and used.

DETAILED DESCRIPTION OF THIS INVENTION

The solubility of a preferred MAS, i.e., FF-MAS, in water is very low, i.e., approximately 20 picogram/ml (corresponding to 2×10^{-5} µg/ml). In ethanol, the solubility of FF-MAS is substantially higher, i.e., approximately 12 mg/ml. According to our preliminary investigations,

the highest solubility of FF-MAS in a mixture of ethanol and water (1:2.5) is approximately 0.4 mg/ml. Several other MASs have a similar low solubility in water.

Surprisingly, it has now been found that a solid composition containing MAS and an additive has a good solubility in water. The additives are components which, when added to MAS, provides a composition which can be used to prepare an aqueous solution containing MAS wherein the concentration of MAS is sufficiently high, preferably above about 1 µg/ml.

Examples of preferred additives are water-soluble proteins such as serum albumin, e.g., human serum albumin (hereinafter designated HSA), optionally in recombinant form, enzymes and phospherglycerider such as phosphatidylethanolamin, phosphatidylcholine, phosphatidylserine, and phosphatidylnositol.

Preferably, the solid compositions have a content of water below about 10 %, preferably below about 5 %, more preferred below about 1 % (weight/weight).

Preferably, the solid compositions have a content of organic solvent below about 10 %, preferably below about 5 %, more preferred below about 1 % (weight/weight).

Preferably, the solid compositions have a content of MAS below about 1 %, preferably below about 0.1 %, more preferred below about 0.05 % (weight/weight).

Preferably, the solid compositions have a content of additive above about 50 %, preferably above about 80 %, even more preferred above about 99 %, and most preferred above about 99.9 %.

Preferably the weight ratio between MAS and the additive is in the range from about 1:10, preferably from about 1:50, to about 1: 5,000. A preferred range is about 1:1000.

Preferred solid compositions are such which can be treated with an aqueous medium containing no or only low concentrations of organic solvent resulting in a solution having a sufficiently high concentration of MAS, e.g., above about 0.001 µg/ml, preferably above about 1 µg/ml. Preferably, these aqueous media contain below about 1 %, preferably below about 0.5 %, more preferred below about 0.1 % of organic solvent (weight/weight).

Earlier, several attempts to prepare compositions fulfilling this requirement have failed.

Herein, the term MAS designates compounds which mediate the meiosis of oocytes. More specifically, MASs are compounds which in the test described in Example 1 below has a per-

centage germinal vesicle breakdown (hereinafter designated GVB) which is significantly higher than the control. Preferred MASs are such having a percentage GVB of at least 50 %, preferably at least 80 %. Examples of preferred MASs are 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol (hereinafter designated FF-MAS); 4,4-dimethyl-5α-cholest-8,14,24-trien-3β-ol hemisuccinate; 5α-cholest-8,14-dien-3β-ol; 5α-cholest-8,14-dien-3β-ol hemisuccinate; (20S)-cholest-5-en-3β,20-diol; 3β-hydroxy-4,4-dimethyl-5α-chola-8,14-dien-24-oic acid-N-(methionine) amide; and cholest-5-en-16β-ol. Further examples of MASs are mentioned in WO 96/00235, 96/27658, 97/00884, 98/28323, 99/58549, 99/67273, 98/52965, 98/55498, 99/32506, WO 00/35938, WO 00/35938, WO 00/53618, and 98/54965, more specifically in Claim 1 thereof and the specific compounds mentioned therein.

Generally, the solid composition is prepared in a manner known per se.

One way of preparing the solid compositions is to prepare a solution of MAS in an organic solvent such as ethanol and, then, to prepare an aqueous solution of the additive. Thereafter, the two solutions are mixed. After mixing the two solutions, the solvent is evaporated or allowed to evaporate. The evaporation can be accelerated by using continuous airflow over the mixed solutions, vacuum drying, freeze drying or any other feasible methods generally known to remove the solvent. Preferably, all these process steps are performed in the absence of oxygen or in the presence of only a minor amount of oxygen. Similarly, the final solid composition is kept in an atmosphere containing no or only a minor amount of oxygen. A preferred way of doing this is to store the solid composition in a closed container wherein the atmosphere has a low content of oxygen, e.g., in nitrogen or argon. These containers may be glass containers or containers of plastic having no undesired action on the solid composition. Preferred examples of such containers are capped vials, capouls, sealed dishes for IVF or IVM treatment or other sealed containers.

The containers of this invention are filled with the atmosphere and the solid composition mentioned in the claims below in a manner known per se.

The product marketed could be a delivery system having one or more depressions or hollows. Hereinafter, these depressions and hollows are mutually designated hollows. At least one of these hollows contains a solid composition and, in the same hollow, the atmosphere has a low content of oxygen, e.g., is nitrogen or argon. A convenient way of placing the solid MAS therein is first to place a solution containing MAS and the additive in the hollow and thereafter to evaporate the solution, preferably in an atmosphere having a low content of

oxygen, e.g., in nitrogen or argon. In this way, the evaporation residue, i.e., the solid composition, is placed directly in the hollow in said device (delivery system).

Since the solid compositions are to be used for the treatment of oocytes, it is important that the solid compositions do not contain constituents that influence the oocytes negatively.

One way of using the solid compositions is to dissolve the composition in an aqueous medium such as water and then, if desired, to add other constituents that may have a favourable influence on the maturation of the oocytes.

Another way of using the composition is to dissolve it in a media normally used for IVF or IVM.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, in any combination thereof, be material for realising the invention in diverse forms thereof.

Example 1

Method used for determining whether a compound is a MAS or not.

Oocytes were obtained from immature female mice (C57BL/6J x DBA/2J F1, Bomholtgaard, Denmark) weighing 13-16 grams, that were kept under controlled temperature (20-22 °C), light (lights on 06.00-18.00) and relative humidity (50-70 %). The mice received an intraperitoneal injection of 0.2 ml gonadotropins (Gonal-F, Serono) containing 20 IU FSH and 48 hours later the animals were killed by cervical dislocation. The ovaries were dissected out and the oocytes were isolated in Hx-medium (see below) under a stereo microscope by manual rupture of the follicles using a pair of 27 gauge needles. Spherical oocytes displaying an intact germinal vesicle (hereinafter designated GV) were divided in cumulus enclosed oocytes (hereinafter designated CEO) and naked oocytes (hereinafter designated NO) and placed in α-minimum essential medium (α-MEM without ribonucleosides, Gibco BRL, Cat. No. 22561) supplemented with 3 mg/ml bovine serum albumin (BSA, Sigma Cat. No. A-

7030), 5 mg/ml human serum albumin (HSA, State Serum Institute, Denmark), 0.23mM pyruvate (Sigma, Cat. No S-8636), 2 mM glutamine (Flow Cat. No. 16-801), 100 IU/ml penicillin and 100 µg/ml streptomycin (Flow, Cat No. 16-700). This medium was supplemented with 3 mM hypoxanthine (Sigma Cat. No. H-9377) and designated Hx-medium.

The oocytes were rinsed three times in Hx-medium and oocytes of uniform size were divided into groups of CEO and NO. CEO and NO were cultured in 4-well multidishes (Nunclon, Denmark) in which each well contained 0.4 ml of Hx-medium and the compound to be tested in a concentration of 10 µM. One control well (i.e., 35-45 oocytes cultured in identical medium with no addition of test compound) was always cultured simultaneously with 3 test wells (35-45 oocytes per well supplemented with test compound).

The oocytes were cultured in a humidified atmosphere of 5 % CO_2 in air for 24 hours at 37°C. By the end of the culture period, the number of oocytes with GV, GVB and polar bodies (hereinafter designated PB), respectively, were counted using a stereo microscope (Wildt, Leica MZ 12). The percentage of GVB, defined as percentage of oocytes undergoing GVB per total number of oocytes in that well, was calculated as:

% GVB = ((number of GVB + number of PB)/ total number of oocytes) X 100.

Example 2

Method used for determining whether a compound can be used as the additive in the solid compositions or not.

An additive for FF-MAS compositions are characterised by:

Improving the solubility of FF-MAS in ethanol/water (1:2.5 v/v)

Ensuring a clear solution of FF-MAS after reconstitution of the composition in MEM Alpha Medium.

Securing percent GVB is at least 50 % preferable 80 % when tested on oocytes obtained from immature female mice.

Prepare a saturated ethanolic solution of FF-MAS. Blend with an aqueous solution of the additive in the ration 1:2.5. By visual inspection control that surplus FF-MAS is available in the solution. Rotate the solution for 24 hours at room temperature. Filter the solution through 0,22 µm filter, determine the content of FF-MAS by HPLC and calculate the solubility. Transfer 350 µl to 4-well dish and evaporate to dryness at room temperature. Add 500 µl MEM ALPHA medium (Gibcobal). If a clear solution is obtained within half an hour, the composition is tested on oocytes obtained from immature female mice. % GVB obtained is at least 50 %, preferable 80 %, vide example 1.

Example 3

Composition containing Human Serum Albumin (HSA).

FF-MAS is dissolved in ethanol (25 µg FF-MAS/ml ethanol). HSA is dissolved in water (1 %). 100 µl of the above mentioned ethanol solution (corresponding to the amount *of* FF-MAS needed for one treatment) and 350 µl of the above mentioned HSA solution were mixed in a glass vial. The resulting mixture of FF-MAS and HSA was evaporated to dryness by airflow of argon. After the liquid was evaporated, the vial was sealed by a rubber septum and the vial was placed at a temperature of 2-8°C. At the time for usage, 500 µl of freshly prepared IVM media was added to the residue in the vial and shaken calmly for 1-2 minutes. The IVM media used was TCM 199 with Earle's salts (Sigma) to which was added 0.8% HSA, 2 mM L-glutamine, 0.25 mM sodium pyrovate, 100 IU/ml penicillin G, and 100 microgram/ml streptomycin. The liquid was transferred to a 4-well dish and the composition was tested on oocytes obtained from immature female mice. Percentage GVB obtained, see below in Table 1. The stability of the formulation, as well as the recovery of FF-MAS from the vials were continously followed, see below in Table 2.

Table 1: **GVB data for FF-MAS in combination with HSA**

Content of HSA in each vial: 6 mg/ml

Substance	Dose FF-MAS (µmol/l)	Number of oocytes	%GVB
Control	-	35	14
FF-MAS*	10	35	43
FF-MAS	0.24	35	86
FF-MAS	0.48	33	88
FF-MAS	1.2	35	91
FF-MAS	2.4	34	97
HSA-control	-	33	9

* internal control dissolved in ethanol

Table 2: **Stability of FF-MAS in combination with HSA**

Content of FF-MAS in each vial: 10 µg/ml

Content of HSA in each vial: 5 mg/ml

Storage Conditions	Months of Storage	Assay µg/ml	% Recovery
5 °C	2 months	10,15	101%
	3 months	10,3	103%
25 °C / 60 %RH	2 months	9,71	97%
	3 months	9,5	95%

"RH" designates relative humidity.

CLAIMS

1. A closed container containing an atmosphere having a low content of oxygen and further containing a solid composition with high aqueous solubility comprising MAS and an additive.

2. A container, according to claim 1, wherein the content of oxygen in the atmosphere is below about 10 %, preferably below about 5 %, more preferred below about 1 % (vol/vol).

3. A container, according to claim 1 or 2, wherein a substantial part of the atmosphere is nitrogen or argon.

4. A container, according to any one of the preceding claims, wherein the content of nitrogen or argon in the atmosphere is above about 90 %, preferably above about 95 %, more preferred above about 99 % (vol/vol).

5. A container, according to any one of the preceding claims, characterised in that the content of water in the solid composition is below about 10 %, preferably below about 5 %, more preferred below about 1 % (weight/weight).

6. A container, according to any one of the preceding claims, characterised in that the content of organic solvent in the solid composition is below about 10 %, preferably below about 5 %, more preferred below about 1 %.

7. A container, according to any one of the preceding claims, characterised in that the content of MAS in the solid composition is below about 10 %, preferably below about 5 %, more preferred below about 2 %, most preferred below about 1 % (weight/weight).

8. A container, according to any one of the preceding claims, characterised in that the MAS is 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol; 4,4-dimethyl-5α-cholest-8,14,24-trien-3β-ol hemisuccinate; 5α-cholest-8,14-dien-3β-ol; 5α-cholest-8,14-dien-3β-ol hemisuccinate; (20S)-cholest-5-en-3β,20-diol; 3β-hydroxy-4,4-dimethyl-5α-chola-8,14-dien-24-oic acid-N-(methionine) amide; and cholest-5-en-16β-ol.

9. A container, according to any one of the preceding claims, characterised in that the additive is a protein or a phosphorglycerid, preferably serum albumin, most preferred human serum albumin.

10. A container, according to any one of the preceding claims, characterised in that the content of additive in the solid compositions is above about 90 %, preferably above about 95 %, even more preferred above about 98 %, and most preferred above about 99 %.

11. A container according to any one of the preceding claims, characterized in that it is a device having one or more hollows among which at least one of the hollows contains an atmosphere with a low content of oxygen and the solid composition.

12. A container, according to any one of the preceding claims, characterised in that the solid composition can be used for preparing an aqueous solution with the characteristics mentioned in any of the following product claims.

13. A container, according to any one of the preceding claims, characterised in that the solid composition can be used for preparing an aqueous solution which when used for the treatment of oocytes can result in a percentage germinal vehicle breakdown (GVB) of at least 50 %, preferably at least 80 %, when MAS is FF-MAS.

14. A container, according to any one of the preceding claims, characterised in that when an aqueous media is added to the solid composition, a solution containing MAS in a concentration of above about 0.001 µg/ml, preferably above about 0.01 µg/ml, more preferred above about 0.1 µg/ml, even more preferred above about 1.0 µg/ml, and most preferred about 10 µg/ml, can be obtained.

15. A container, according to any one of the preceding claims, characterised in that when an aqueous media is added to the solid composition, a solution containing MAS in a concentration of below about 0.1 g/ml, preferably below about 0.01 g/ml, can be obtained.

16. A container, according to any one of the preceding claims, characterised in that when water is added to the solid composition, an aqueous solution wherein the content of or-

ganic solvent is below about 0.1 %, preferably below about 0.05 %, most preferred below about 0.01 %, can be obtained.

17. A process for preparing a closed container containing an atmosphere having a low content of oxygen and further containing a solid composition comprising MAS and an additive, characterized by:

    a) preparing a solid composition comprising MAS and an additive,

    b) filing the solid composition in a container,

    c) before, during or after step b), filling the container with an atmosphere having a low content of oxygen, and

    d) closing the container.

18. A process, according to the previous claim, wherein the preparation of the solid composition comprising MAS and an additive is performed under conditions where there is a low concentration of oxygen, preferably in an atmosphere having a low content of oxygen.

19. A process, according to any of the previous process claims, characterized in that the container prepared has the characteristics mentioned in any of the above product claims.

20. Any novel feature or combination of features described herein.

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ABSTRACT

A solid, stable composition containing a meiosis activating substance can be prepared by adding a protein or a phosperglycid in the presence of an atmosphere having a low content of oxygen.